

Characterization of Parrot Bornavirus-4 Infection in Immortalized and Primary Cell Lines

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Abstract

Parrot bornavirus 4 (PaBV-4) remains poorly characterized with respect to the cellular determinants of its host range, genetic adaptation, and interactions with related orthobornaviruses. Here, we performed an in-depth *in vitro* characterization of PaBV-4 by evaluating its replication kinetics in avian and mammalian cell cultures, whole-genome sequencing during serial passage, and virus interference studies. Replication kinetics showed that PaBV-4 could establish persistent infection in most avian but no mammalian cell lines, with highest magnitude of propagation in quail fibrosarcoma (QT-35) and duck embryo (CCL-141) fibroblasts, although turkey embryo fibroblasts were not permissive. Sequencing of viral genomes from QT-35, CCL-141, and DF-1 (immortalized chicken fibroblasts) after ~ 24 passages showed only limited genetic diversification, with no evidence of host-specific molecular adaptation despite differences in cellular permissivity, indicating PaBV-4 genomic stability. QT-35, CCL-141, and DF-1 that were persistently infected with PaBV-4 or aquatic bird bornavirus-1 (ABBV-1; a related orthobornavirus) completely inhibited establishment of secondary infection with the other virus, whereas simultaneous coinfection favored PaBV-4 compared to ABBV-1 replication. In contrast, only transient inhibition of Newcastle disease virus (NDV; a distantly related mononegavirus) replication was observed in PaBV-4-persistently infected cells. Collectively, these findings demonstrate that cellular permissivity, rather than rapid viral adaptation, is the principal determinant of PaBV-4 replication *in vitro* and demonstrate strong viral interference among different orthobornavirus species.

Introduction

Parrot Bornavirus-4 (PaBV-4) is an enveloped virus with a negative sense, single-stranded and non-segmented RNA genome classified in the *Mononegavirales* order, *Orthobornavirus* genus, and *Bornaviridae* family. This virus causes a chronic, non-lytic infection, characterized by nuclear replication and persistence^{1,2}. Together with other parrot bornaviruses (PaBVs), PaBV-4 is one of the recognized etiological agents of proventricular dilatation disease (PDD), a fatal and progressive disease predominantly affecting captive psittacines (i.e., parrots and allies), characterized by inflammation of the central and peripheral nervous systems^{1,3}. Although psittacines are among the most frequently affected, experimental and anecdotal evidence indicate that PaBV-4 can display a broad host range across multiple avian taxa, as suggested by natural infection of birds taxonomically distant from psittacine, such as galliformes (i.e., the Himalayan monal, *Lophophorus impejanus*)⁴.

In vitro studies have demonstrated that PaBV-4 can infect and establish persistent infection in cells originating from chicken, quail, and duck (CEC-32, QT-6, QM-7, LMH, DF-1, and DEF), while mammalian cells (including Vero, OL, COS-7, BHK, MDCK, and C6) are nonpermissive⁵⁻⁷. Given the ability of PaBV-4 to infect cell lines from multiple avian species, it remains unclear whether differences in the efficiency of viral propagation among permissive cell lines could offer insights into the varying susceptibility to infection of the originating species. While propagation in cell lines may not be sufficiently predictive to resolve subtle differences in host susceptibility, it has the potential to reveal major differences in cellular

permissiveness between distant taxonomic groups^{8,9}. Specifically for orthobornaviruses, the nuclear localization signal (NLS) of the nucleoprotein (N) has been shown to be an important determinant of host range restriction between mammalian and avian species, as chimeric borna disease virus-1 (BoDV-1, a mammalian orthobornavirus and type species of the *Bornaviridae* family) expressing the NLS sequence of PaBV-4 has been shown to have a diminished capacity to replicate in mammalian cell lines when compared to wildtype BoDV-1 controls⁵. Additionally, it remains unknown whether specific mutations in the viral genome exist that may promote replicative fitness in certain avian hosts or cell culture environment. While single nucleotide polymorphisms are expected to arise in cell culture, as established for other viruses^{10,11}, understanding whether orthobornaviruses can become adapted to *in vitro* propagation is relevant, as large cell culture preparations (resulting in many passages) are needed to provide sufficient virus for either *in vitro* or *in vivo* studies, due to the low yield of these viruses in the supernatant of infected cells^{12,13}.

Virus superinfection is the infection of a cell, tissue, or organism by a second (i.e., superinfecting) virus after an initial viral infection has already been established. Viral interference occurs when the previous virus infection inhibits or prevents infection or replication of the superinfecting virus within the same cell, tissue, or host.^{14,15} As persistent, chronic infection with orthobornaviruses can last for weeks to years without development of clear cytopathic effect or tissue damage (at least in carrier species)^{1,16}, infection with other viruses is likely to occur during this extended timeframe. Interestingly, however, orthobornaviruses appear to exert marked virus exclusion. For instance, transgenic mice constitutively expressing BoDV-1 N protein in neurons are resistant to BoDV-1 infection¹⁷, while Vero cell persistently infected with BoDV-1 prototype He/80 are resistant to superinfection with the V strain¹⁸. This superinfection exclusion, however, does not appear to occur between high- and low-fitness BoDV-2 variants *in vitro*¹⁹, a mechanism that could promote genetic diversity and improve long term adaptability of the virus. While natural coinfection with orthobornaviruses in mammals has not been reported, among avian species there is evidence of mixed infection of single psittacine birds with PaBV-2 and PaBV-4, PaBV-2 and PaBV-6, or PaBV-4 and PaBV-7 (reviewed by Rubbenstroth et al.¹). Of note, cockatiels persistently infected with PaBV-4 and experimentally infected with PaBV-2 developed more severe disease than those infected with PaBV-2 alone. While the coinfection was not an intended parameter of that study (inadvertent PaBV-4 infection was discovered only later in two birds), the authors suggest that coinfection is not only possible, but may influence pathogenesis and exacerbate disease²⁰.

Based on the gap of knowledge outlined above, the objectives of this study were to: 1) further characterize the host range of PaBV-4 by assessing its differential growth in immortalized and primary cell lines derived from mammalian and avian species; 2) to describe any genomic mutations associated with adaptation to different species or *in vitro* propagation; and 3) to document any superinfection resistance to infection with aquatic bird bornavirus-1 (ABBV-1), used a closely related virus within the same family, and lentogenic Newcastle disease virus (NDV), used as distant representative in the same order.

Results

Propagation of PaBV-4 in immortalized duck fibroblasts and virus stock production

The PaBV-4 was isolated from the pooled tissues of a naturally infected rose-breasted parakeet (*Psittacula alexandri*) into primary duck embryo fibroblasts (DEF)²¹, and then propagated in immortalized duck embryo fibroblasts (CCL-141). These cells were scaled to obtain ~ 24 ml of clarified cell lysate that was concentrated with polyethylene glycol (PEG) to obtain a final virus concentration of 2.18×10^3 focus-forming units (FFU) / mL, as assessed by immunofluorescence (IFA). This stock was used for all the other downstream applications. Infected CCL-141 displayed a characteristic IFA reactivity pattern against the N protein, which was predominately nuclear and finely stippled, with a lower frequency of cytoplasmic reactivity (**Fig. S1a**), while no signal was seen in uninfected control cells (**Fig. S1c**). Neither infected (**Fig. S1b**) nor uninfected (**Fig. S1d**) cells showed cytopathic effect (CPE). Infection was further confirmed by RT-PCR: total cellular RNA was extracted from infected CCL-141, and reverse-transcription PCR used to amplify a portion of the N gene, which yielded a band of approximately 200 bp on gel electrophoresis (data not shown), as expected^{12,22}. Sanger sequencing of the band (Advanced Analysis Centre Genomics Facility, University of Guelph, Ontario, Canada) confirmed 100% identity with the published UoG-PK sequence (Accession No MZ018602). The identity of the virus was further confirmed by whole genome sequencing (see below).

Immortalized quail and duck fibroblasts support the highest levels of PaBV-4 replication

The virus stock produced in CCL-14 was used to assess the replication kinetics of PaBV-4 in six immortalized and four primary cell lines from both avian and mammalian species. Cells were infected at a multiplicity of infection (MOI) of 0.001, and passaged every three days for a total of six passages (18 days post-infection; dpi), with virus growth estimated at every passage (**Fig. S2**).

PaBV-4 N Protein Expression in Avian and Mammalian Cell Lines. The percentage of cells immunoreactive for PaBV-4 was estimated by IFA for the N protein (Fig. 1a-c). Of the 6 immortalized cell lines tested (Fig. 1a), PaBV-4 replication proved most efficient in CCL-141 (Fig. 1d, g) and immortalized quail fibrosarcoma cells (Fig. 1e, h), in which most cells displayed stippled intra-nuclear immunoreactivity by passage 4 post-infection (12 dpi). In these cells, PaBV-4 replication was initially slow, with few positive cells at passage one (3 dpi); however, by passage 2 (6 dpi) and 3 (9 dpi) on average more than 20% of CCL-141 and QT-35 cells, respectively, showed immunoreactivity. Although PaBV-4 could infect immortalized chicken embryo fibroblasts (DF-1), replication kinetics were delayed compared to QT-34 and CCL-141, with an average of only ~ 12% of cells displaying immunoreactivity by passage 6 (endpoint; Fig. 1f, i). PaBV-4 replication did not occur in any of the mammalian cell lines (Vero [African green monkey kidney epithelial] CT-2A [mouse glioma], BHK-21 [baby hamster kidney]), as shown by lack of IFA

signal at every passage (Fig. 1j, m). Of the 4 primary avian cell lines (Fig. 1b), some degree of immunoreactivity, indicating infection, was observed in chicken, duck and Canada goose embryo fibroblasts (respectively CEF, DEF, and cGEF), albeit the percentage of immunoreactive cells increased at a slower rate and was overall lower compared to CCL-141 and QT-35 by the end of the study. In CEF, immunoreactivity was first detected in ~ 1.5% of cells at passage 5, and reached ~ 6.5% by passage 6, suggesting PaBV-4 can replicate in CEF, although less efficiently than in cGEF and DEF (Fig. 1k, n), which displayed a higher increase in the average proportion of immunoreactive cells between passage 2 and 3 (6% to 21.7%), and 4 and 5 (9.2% to 21.7%), respectively. Turkey embryo fibroblasts showed no reactivity (TEF; Fig. 1l, o).

Integration of the proportion of immunoreactivity over the entire course of the experiment to generate empirical areas under the curve (eAUC; Fig. 1c) revealed marked differences across cell lines. CCL-141 exhibited the greatest cumulative proportion of PaBV-4-immunoreactive cells, differing significantly from all other cell lines, including QT-35 ($p < 0.0001$). QT-35 formed the second highest group and exceeded all remaining cell lines ($p < 0.0001$) except cGEF ($p = 0.124$). Among the primary avian cultures, cGEF exhibited greater cumulative immunoreactivity than DEF, CEF, TEF, and the mammalian cell lines ($p \leq 0.0015$), whereas DEF showed greater cumulative immunoreactivity than CEF, TEF, and the mammalian cell lines ($p \leq 0.0175$). No significant differences were detected between DF-1, CEF, TEF, and the mammalian cells ($p > 0.05$; one-way ANOVA with Tukey's test for multiple comparisons).

PaBV-4 RNA Accumulation in Avian and Mammalian Cell Lines. Viral RNA copy number per 100 ng of total cellular RNA was quantified in triplicate at each passage, normalized to the value measured at passage zero (baseline), and $\log_{10}$ transformed. In the immortalized avian cell lines (Fig. 2a), the greatest increase in virus RNA copies was observed in QT-35 and CCL-141, which peaked at passage 5 with a mean $\log_{10}(\text{FC})$ value of 4.68 and 3.63, respectively. An increase in RNA virus copies was observed in DF-1 cells as well, although at a diminished rate and magnitude when compared to the other two immortalized avian cell lines tested, with the highest average value ($2.65 \log_{10}(\text{FC})$) recorded at passage 6. There was no increase in N gene copies among the selected mammalian cell lines, and virus copy number decreased over the course of the experiment, indicating that PaBV-4 genetic material was gradually cleared. In the primary cells (Fig. 2b), the greatest absolute RNA fold change was seen in DEF and CEF ($2.39 \log_{10}(\text{FC})$ and $1.73 \log_{10}(\text{FC})$) at passage 6, and cGEF ($2.32 \log_{10}(\text{FC})$) at passage 4. Virus RNA copies in TEF plateaued at passage three ($0.75 \log_{10}(\text{FC})$), and decreased from passage 4 onwards, averaging to $0.23 \log_{10}(\text{FC})$ by passage 6, an increase of only 1.7% over the baseline.

Integration of the RNA FC values over the entire course of the experiment to generate empirical areas under the curve (eAUC; Fig. 2c) showed marked differences in the cumulative ability of PaBV-4 to amplify its genome across cell lines. QT-35 exhibited the highest cumulative viral RNA accumulation (eAUC), differing significantly from all other cell lines, including CCL-141 ($p = 0.0007$). CCL-141 formed a second distinct group, exceeding all remaining avian and mammalian cell lines ($p < 0.0001$). DF-1 exhibited intermediate RNA accumulation, exceeding all remaining cell lines, with the smallest difference observed

relative to cGEF ($p = 0.0396$). Among primary avian cultures, cGEF supported greater RNA accumulation than CEF and TEF ($P \leq 0.0003$), whereas CEF, TEF, and DEF did not differ significantly. The three mammalian cell lines exhibited the lowest RNA accumulation, did not differ from one another, and were significantly lower than all avian cell lines ($p \leq 0.0205$; one-way ANOVA with Tukey's test for multiple comparisons). Overall, the accumulation of PaBV-4 RNA largely paralleled the eAUC values obtained from IFA, indicating broad agreement between viral RNA amplification and N protein expression. However, several avian cell lines, particularly DF-1, exhibited measurable increases in viral RNA despite limited IFA reactivity.

Limited genetic diversification of PaBV-4 during propagation in avian cell lines

To assess the possible development of mutation during virus isolation and multiple passages (p) in cells of different species, the full genome of PaBV-4 was sequenced from the original infected tissues, during virus isolation (DEF-p5), stock production (CCL-141-p15), and after growth curves completion (DF-1-p21, QT-35-p21, and CCL-141-p24; see Methods for details). The virus genome sequenced from tissues was used as the reference for comparison.

The full-length of the genome could be sequenced in all samples, showing a full length of 8,915 nt. Considering all the cells lines and passages, 51 nucleotide substitutions were identified at 24 unique genomic positions (Fig. 3, and **Table S1**). Of these, 20 occurred within coding regions and 4 in non-coding regions. Changes in 5 genomic positions occurred within overlapping open reading frames, resulting in amino acid or codon (for synonymous mutations) changes in two different proteins. Most coding mutations (19 / 25; 76%) were missense substitutions, whereas the remaining six (6 / 25; 24%) were synonymous.

The most frequently detected mutation (7 / 51) was a C > T transition at genomic position 7207, resulting in a leucine to phenylalanine substitution at residue 653 of the polymerase (L) protein. This variant was identified in every sequenced sample, from the original DEF-p5 isolate through all subsequent passages, and ranged from 10.55 to 61.45% of sequencing reads. The second most common (6 / 51) mutation occurred at position 6935 (T > C), resulting in an isoleucine to threonine substitution in the L protein. This mutation was detected in all propagated cell lines except DEF-p5, with frequencies ranging from 6.39 to 20.75% of reads. Additional recurrent missense mutations were identified in the phosphoprotein (P), glycoprotein (G), and L proteins, although these generally occurred at lower frequencies and in fewer samples (Fig. 3; **Table S1**). Most remaining missense mutations were detected only in individual samples and at low frequencies (5–17% of reads). Synonymous substitutions were less common (6 / 25 coding changes; 24%) and primarily affected the P, matrix (M), and L proteins. Four nucleotide substitutions were identified in non-coding regions, of which only the T > C transition at position 1190 recurred consistently, increasing to frequencies of 40.74–75.17% following serial passage

in CCL-141 and DF-1 cells. The remaining non-coding variants were each detected only in one cell passage and at low frequencies (Fig. 3; **Table S1**).

Using the tissue-derived PaBV-4 consensus genome as the ancestral reference, the apparent accumulation of nucleotide substitutions was estimated independently for each propagated lineage. Two substitutions became detectable during the initial propagation in DEF cells (p5), followed by six additional substitutions after continued passage in CCL-141 cells (p15). Subsequent propagation resulted in three newly detected substitutions in CCL-141 p24, nine and six substitutions in DF-1 p21A and p21B, respectively, no additional substitutions in QT-35 p21A, and four substitutions in QT-35 p21B (Table 1). These nucleotide changes corresponded to an apparent substitution accumulation rate ranging from 4.3 to 9.1×10^{-5} substitutions per nucleotide per passage (mean, 6.5×10^{-5}).

Table 1
Apparent substitution accumulation rates during serial passage of PaBV-4 in cell lines.

Terminal lineage	Total passages	Cumulative newly detected substitutions	Apparent substitution accumulation rate (substitutions/site/passage)
CCL-141 p24	24	11	5.14×10^{-5}
DF-1 p21A	21	17	9.08×10^{-5}
DF-1 p21B	21	14	7.48×10^{-5}
QT-35 p21A	21	8	4.27×10^{-5}
QT-35 p21B	21	12	6.41×10^{-5}
Mean	-	-	6.48×10^{-5}
Apparent substitution accumulation rates were calculated independently for each terminal lineage. Newly detected substitutions were identified sequentially relative to the immediately preceding ancestral population (tissue-derived virus → DEF p5 → CCL-141 p15 → terminal lineage). Only newly detected substitutions were counted. Rates were calculated by dividing the cumulative number of newly detected substitutions by the product of the PaBV-4 genome length (8915 nucleotides) and the total number of passages elapsed since the tissue-derived virus.			

Reciprocal viral interference prevents superinfection by PaBV-4 and ABBV-

To assess whether PaBV-4 and ABBV-1 exhibit reciprocal viral interference *in vitro*, naïve QT-35 and QT-35 persistently infected with PaBV-4 or ABBV-1 were challenged (MOI = 0.01) with each virus. Moreover, naïve QT-35 were infected simultaneously with the same amount of infectious PaBV-4 and ABBV-1 (each, MOI = 0.01). The concentration of each virus RNA was evaluated over 45 dpi, corresponding to ~ 11 passages, by RT-qPCR (RNA copies from total cellular RNA) and RNAscope *in situ* hybridization. To

assess the fold changes compared to the beginning of the study, the magnitude of RNA copies at each time was normalized to 3 dpi.

In naïve QT-35 cells, both ABBV-1 and PaBV-4 established productive persistent infections, with viral RNA increasing progressively throughout the 45-day observation period. By the end of the study, both ABBV-1 and PaBV-4 RNA increased approximately 120-fold relative to the baseline at 3 dpi (Fig. 4a, c; purple line). Simultaneous coinfection also resulted in progressive accumulation of viral RNA for both viruses, although the amount of ABBV-1 RNA was severely reduced compared to the corresponding single-virus infection, while PaBV-4 RNA was not and comparable to naïve infected cells (Fig. 4a, c; black line). In contrast, reciprocal superinfection of persistently infected cells markedly inhibited establishment of persistent infection by the second virus. ABBV-1 RNA remained near baseline when QT-35 cells persistently infected with PaBV-4 were challenged with ABBV-1, with only a transient increase observed at 21 dpi (Fig. 4a, green line). Similarly, PaBV-4 RNA failed to increase following infection of ABBV-1-persistently infected cells (Fig. 4c, orange line). The RNA levels of the resident viruses fluctuated only mildly around the baseline, although both ABBV-1 (Fig. 4a, orange line) and PaBV-4 (Fig. 4c, green line) had a temporary increase (~ 4–10-fold change) at 21 and 17 dpi, respectively.

Viral RNA copies were integrated over the entire course of the experiment to generate empirical areas under the curve (eAUC; Fig. 4b, d). When ABBV-1 RNA was tested (Fig. 4b), eAUC was highest in persistently ABBV-1-infected cells challenged with PaBV-4 (as expected, as these cells were already persistently infected at the beginning of the study), intermediate in cells infected with ABBV-1 at the beginning of the study, and significantly reduced during simultaneous coinfection or in cells that were persistently infected with PaBV-4 and challenged with ABBV-1. When PaBV-4 RNA was tested (Fig. 4d), the highest eAUC was observed in cells persistently infected with PaBV-4 and challenged with ABBV-1, and the lowest eAUC was seen when cells persistently infected with ABBV-1 were challenged with PaBV-4, mirroring what seen for ABBV-1. However, simultaneous coinfection yielded cumulative PaBV-4 RNA levels comparable to those of PaBV-4 single infection, a finding markedly different to what seen with ABBV-1. Collectively, these findings indicate that both viruses are markedly susceptible to interference when a previous orthobornavirus infection is already established. However, under simultaneous infection conditions, PaBV-4 exhibited greater cumulative RNA accumulation than ABBV-1, suggesting that it may possess a competitive replicative advantage during co-infection.

RNAscope analysis corroborated the RT-qPCR findings. By the end of the experiment, there was widespread ABBV-1 RNA signal (> 70% of cells) in QT-35 that were persistently infected with ABBV-1 (Fig. 5a, b; red probe). There was no reactivity for PaBV-4 in cells that were not superinfected (Fig. 5c; negative control), while in the PaBV-4 superinfection group only rare cells were reactive (Fig. 5d; blue signal). Similarly, PaBV-4-persistently infected QT-35 exhibited abundant (approximately 40–70%) PaBV-4 RNA signal (Fig. 5e, f; blue signal), while no ABBV-1 RNA was detected in the negative control cells (Fig. 5g), and only rarely in the ABBV-1 superinfection group (Fig. 5h; red signal). When QT-35 were simultaneously infected, both PaBV-4- and ABBV-1-positive cells could be observed, each accounting for

approximately 70 and 5–10% of cells (Fig. 5i), respectively. RNAscope signal for both viruses was finely dispersed and cytoplasmic, with no reactivity in negative cells (Fig. 5J).

PaBV-4 persistence does not inhibit NDV infection

To determine whether persistent infection with PaBV-4 is associated with the development of superinfection resistance against another virus in the *Mononegavirales* order, CCL-141, DF-1 and QT-35 persistently infected with PaBV-4 were challenged with an NDV strain expressing the red fluorescent protein (RFP; NDV-RFP) at MOI = 1 or 0.01. Supernatant samples were collected at 0, 6, 24, 30 and 48 hours post-infection (hpi; MOI = 0.01), and 0, 6, 24, and 30 hpi (MOI = 1), and infectious NDV-RFP titered in DF-1 cells by limiting dilutions. Another set of cells infected at MOI = 0.01 was used for total cellular RNA extraction at 6 and 24 hpi, to estimate the RNA copies of PaBV-4 and NDV-RFP. Cells negative for PaBV-4 and infected with NDV-RFP served as controls.

At an MOI of 0.01, PaBV-4-infected QT35 yielded significantly lower NDV-RFP titers in the supernatant at 24 and 30 hpi, compared to control cells ($p < 0.05$; two-way ANOVA with Sidak's multiple comparisons test; Fig. 6a), while at an MOI of 1, significantly different titers were observed only at 24 hpi ($p = 0.0340$; Fig. 6b). Titer differentials were never greater than 2 $\log_{10}$ units. In CCL-141, infectious titres of NDV-RFP were significantly lower in PaBV-4-infected cells at 30 ($p = 0.0269$) and 48 ($p = 0.0259$) hpi at an MOI of 0.01, and 30 hpi ($p = 0.0463$) at an MOI of 1, with the differential always less than one $\log_{10}$ unit. In DF-1, at either MOI, no significant differences were seen between double infected and controls ($p > 0.05$; two-way ANOVA with Sidak's multiple comparisons test). When the growth of NDV-RFP was assessed using eAUC across all time points, no differences were observed between cell lines that were or not persistently infected with PaBV-4 (one-way ANOVA with Tukey's test for multiple comparisons; data not shown).

When virus RNA was tested, at 6 hpi, no significantly different amounts of NDV-RFP RNA copies were measured between persistently infected and control cells. At 21 hpi, the concentration of NDV-RFP RNA copies was significantly lower in QT-35 ($p < 0.0001$) and CCL-141 ($p = 0.0020$) persistently infected with PaBV-4 compared to the controls (two-way ANOVA with Sidak's multiple comparisons test; Fig. 6c), a finding consistent with the lower infectious titer observed in the supernatant of QT-35 at 24 and 30 hpi. The concentration of PaBV-4 RNA copies remained nearly identical between cells and between 6 and 21 hpi, suggesting a stable PaBV-4 infection over the course of the experiment (Fig. 6d).

Differences in cell morphology between single and double infected groups were assessed by brightfield microscopy imaging at 0, 18, and 48 hpi. Infected cells developed syncytia formation and severe disruption of the monolayer, regardless of the PaBV-4 status, consistent with only transient decrease of NDV-RFP titers and RNA levels (data not shown).

Discussion

In this study, 10 immortalized and primary cell lines of avian and mammalian origin were tested for their permissivity to support the replication of a PaBV-4 strain, which was initially isolated from the liver and kidney of a rose-breasted parakeet (*Psittacula alexandri*)²². Consistent with previous findings in the literature⁵⁻⁷, we confirmed that mammalian cells (Vero, CT-2A, and BHK-21) are not permissive to PaBV-4 infection, as shown by lack of viral antigen and steady reduction of virus RNA over 6 passages. Conversely, all primary and immortalized cell lines derived from gallinaceous or anatid species, except TEF, supported PaBV-4 propagation, albeit to differing extents. When considering the eAUCs derived from IFA, propagation was best supported, in order, by CCL-141, QT-35, cGEF, DEF, and DF-1. When considering RNA, replication/transcription appeared to be best supported, in order, by QT-35, CCL-141, DF-1, cGEF, DEF, CEF, and TEF. Overall, the cumulative production of viral RNA broadly paralleled the eAUC values derived from IFA, indicating that viral RNA amplification was generally associated with N protein accumulation. However, several avian cell lines, most notably DF-1, had substantial amounts of viral RNA despite exhibiting comparatively limited IFA signal. This suggests that increase in viral RNA can occur in the absence of proportional amounts of detectable viral protein. One possible explanation is reduced translational efficiency of viral mRNAs in certain cell types, although other post-transcriptional mechanisms, including altered viral protein stability, may also contribute²³. A similar finding was observed with ABBV-1, as viral N protein could not be detected by IFA in DF-1 cells despite detection of viral RNA over multiple passages²². Lastly, while increasing viral RNA and N protein expression are consistent with productive infection, they do not directly demonstrate generation of infectious progeny, warranting cautious interpretation of our findings.

When considering both IFA signal and RNA accumulation, replication appeared more efficient in CCL-141 and QT-35 compared to any other avian primary cell line, including DEF, which is most often reported in the literature for the purpose of virus isolation or stock production of avian orthobornaviruses^{12,22}. Our findings support the use of CCL-141 and QT-35 as producer cells for the purpose of PaBV-4 stock generation, although infectious titers should be quantified to confidently make this assessment. Indeed, another quail cell line similar to QT-35 (QT-6) efficiently supported rescue of recombinant PaBV-4 and ABBV-1, while DF-1 did not allow rescue of PaBV-4.^{24,25} The greater ability of immortalized, compared to primary, avian cells to support PaBV-4 replication may be explained by differences in the cell cycle regulation. As the ribonucleoprotein (RNP) of orthobornaviruses is closely associated with the chromosomal histones (a mechanism that allows the RNPs to become segregated into daughter cells during mitosis²⁶), immortalized cell may allow faster virus spread due to shorter doubling times. Additionally, as double-strand breaks in DNA have been shown to be important docking sites for BoDV-1 nuclear persistence and replication²⁷, immortalized cells may feature greater numbers of such scaffolding areas due to increased chromosomal instability and decreased DNA repair ability²⁸. Lastly, immortalized cell lines often exhibit defects in the induction of type I interferon and interferon-stimulated genes, resulting in impaired innate antiviral responses and a more permissive environment for viral replication²⁹.

Notably, PaBV-4 was able to replicate in both primary and immortalized cell lines derived from chickens, CEF and DF-1, respectively, as demonstrated by faint IFA signal and a steady increase in virus RNA copies over the course of the experiment. To the authors' knowledge, this is the first study demonstrating PaBV-4 successful replication in CEF^{30,31}. However, while a certain degree of permissivity to PaBV-4 infection was identified in chicken-derived cells, TEF (derived from turkey, another gallinaceous species) did not show immunoreactivity for PaBV-4 N protein, and only low-level virus RNA copies over the course of the study (1.7% increase compared to the baseline).

After the first round of infection, spread of PaBV-4 in the cell monolayer occurs at least in part through separation of viral material into daughter cells. These findings suggest that the efficiency of viral entry, which is presumably mediated by an as yet unidentified cellular receptor for orthobornaviruses¹, is unlikely to be the primary determinant of the differences in viral propagation observed among cell types. Instead, post-entry host factors are likely to play a greater role in determining cellular permissivity. Indeed, while the lack of replication in mammalian-origin species appeared stark (and reproducible), recent studies suggest that this restriction is not mediated by surface receptor, but rather the ability of the nuclear localization signal (NLS) of the N protein to be recognized by the host cell⁵.

Ultimately, the differences in permissivity observed in this study are likely to reflect intrinsic properties of the cells themselves (e.g., tissue of origin, differentiation state, immortalization history, innate antiviral response), rather than species-specific susceptibility alone. Consequently, extrapolation of these findings to host susceptibility *in vivo* should be made with caution, as interpretation is further limited by the absence of a psittacine-derived cell line, precluding comparison with the putative natural host of PaBV-4¹. The use of continuous cell lines is likely to model only broad host barriers, as illustrated by the marked distinction in permissivity between avian- and mammalian-derived cells observed in the present study. Among the avian cell lines, TEF was the only one that did not support PaBV-4 propagation. Interestingly, another avian orthobornavirus, ABBV-1, has previously been shown to replicate in TEF³² and successfully infect turkeys³³, suggesting that the inability of PaBV-4 to replicate in this cell line may reflect genuine differences in host range between these closely related viruses. However, confirmation of this hypothesis will require *in vivo* studies.

One of the aims of this study was to evaluate the extent of genetic drift in PaBV-4 genome, through whole-genome sequencing at different stages of *in vitro* passaging since the time of isolation from tissues. Serial propagation in diverse avian cell lines over 6–9 passages resulted in relatively little genetic diversification of the PaBV-4 genome. A total of 51 nucleotide substitutions were identified at 24 unique genomic positions; however, no mutation consistently segregated with a particular host species or cell line, suggesting that extensive host-specific adaptation did not occur during the time frame examined. The most recurrent nonsynonymous substitution occurred at nucleotide 7207 in the L gene, resulting in a leucine to phenylalanine substitution in the viral RNA-dependent RNA polymerase. This variant was detected in all permissive cell lines and repeatedly increased to frequencies exceeding 50% of sequence reads, suggesting that it may confer a selective advantage during propagation in cell

culture. Whether this reflects the emergence of a *de novo* adaptive mutation or the selection of a pre-existing low-frequency variant cannot be determined from the present study. Overall, the presence of numerous low-frequency variants is consistent with PaBV-4 existing as a genetically heterogeneous viral population (quasispecies).

The apparent substitution accumulation rate estimated in the present study (mean: 6.5×10^{-5} substitutions / site / passage) further supports the relative genetic stability of PaBV-4 during serial *in vitro* propagation. This should not be interpreted as the intrinsic viral mutation rate, as the actual number of virus replication cycles per cell passage or unit of time remains unknown. Moreover, the apparent substitution rate presented here reflects the combined influence of mutation, selection, genetic drift, and the constraints derived from the variant-calling threshold. Nevertheless, the relatively limited number of newly detected substitutions across independent lineages suggests that host-specific permissivity is largely determined by cellular factors rather than rapid viral genetic adaptation, although it was interesting to note that DF-1 (the least permissive cell line of the three tested) appeared to have increased substitution rates compared to QT-35 and CCL-141. Whether this reflects increased selective pressure in a relatively restrictive cellular environment or simply stochastic genetic drift is unknown. Although the mutation rate of PaBV-4 remains undefined, related viruses such as BoDV-1 also exhibit low mutation rates, estimated at 1.06×10^{-4} substitutions / site / year, which falls within the lower range typically observed for RNA viruses (i.e., 10^{-3} to 10^{-3} substitutions / site / year)³⁴. If our data were to be annualized (assuming 4.5 days between passages), the apparent mutation rates identified in this study would be much higher, spanning $\sim 4.7\text{--}5.9 \times 10^{-3}$ substitutions / site / year. This would be, however, a highly artifactual extrapolation, as it would assume constant cell passaging over a year, a scenario that is unlikely to occur *in vivo*, where orthobornaviruses establish persistent infections primarily in long-lived, terminally differentiated neurons¹.

Another objective of this study was to investigate the phenomenon of interference in cells persistently infected with PaBV-4, using ABBV-1 and NDV-RFP, respectively chosen as taxonomically close (congeneric) or distant interfering viruses. Viral interference has previously been described among orthobornaviruses, where persistent infection with one BoDV-1 strain reduces susceptibility to superinfection by another strain and is thought to contribute to viral persistence and the remarkable genetic stability of this virus group¹⁸. Consistent with these observations, our reciprocal superinfection experiments demonstrated that persistent infection with either PaBV-4 or ABBV-1 markedly reduced RNA accumulation of the superinfecting orthobornavirus (with no effects on the resident virus), indicating that viral interference is bidirectional and extends across avian orthobornavirus species. Interestingly, simultaneous coinfection partially overcame this exclusion, although PaBV-4 consistently achieved greater cumulative RNA levels than ABBV-1, suggesting an intrinsic competitive advantage of PaBV-4 when both viruses establish infection concurrently. The RT-qPCR findings were corroborated by RNAscope *in situ* hybridization, which enabled simultaneous discrimination between PaBV-4 and ABBV-1 RNA, a distinction not currently possible using the IFA assay in our laboratory. Interestingly, once infection had reached a steady state, only a subset of cells within the persistently infected monolayers

showed detectable viral RNA. Despite this, the superinfecting virus failed to establish infection in cells lacking signal for the resident virus. This observation indicates that susceptibility to superinfection is not solely determined by the presence of viral RNA within individual cells, but may instead reflect heterogeneous cellular states or paracrine antiviral mechanisms that extend protection across the monolayer. Such cell-to-cell differences in susceptibility have been increasingly recognized, even among clonally derived cell populations, where stochastic variation in cellular state and antiviral signaling can influence infection outcomes^{35,36}. One limitation of this study is that duplex RNAscope could not be consistently applied to persistently infected and challenged cultures (only in simultaneous; see Methods). Consequently, the two viruses were evaluated on consecutive serial sections, precluding definitive assessment of whether the rare cells containing RNA from the challenge virus also harbored the resident virus. Thus, although our findings demonstrate that establishment of the superinfecting virus was markedly restricted at the population level, they cannot determine whether the occasional challenge-virus-positive cells represented true superinfection of persistently infected cells or - most likely - infection of cells lacking detectable resident virus. Future studies using different probe sets (e.g., fluorescent ones) or recombinant reporter orthobornaviruses are needed to better assess co-infection at the cellular level. Lastly, although simultaneous coinfection resulted in the establishment of cell cultures containing both PaBV-4 and ABBV-1 up to 45 dpi, it unclear whether both viruses would have persisted indefinitely or whether the competitive advantage of PaBV-4 would have ultimately resulted in loss of ABBV-1. Still, the ability of both orthobornaviruses to establish persistent infection following simultaneous exposure raises the possibility that mixed orthobornavirus infections may occur, consistent with reports of mixed infection with different PaBV strains in psittacines¹. Similar, albeit less pronounced, interference was also observed following superinfection with NDV-RFP. QT-35 and CCL-141 persistently infected with PaBV-4 exhibited reduced NDV-RFP titres and lower NDV RNA levels during the early stages of infection. However, these differences were not maintained throughout the experiment, and integration of NDV replication kinetics over time revealed no overall reduction in cumulative virus replication.

Together, these observations suggest that PaBV-4-mediated viral interference is most effective against closely related orthobornaviruses, while exerting only a modest and transient inhibitory effect on a distantly related mononegavirus. The underlying mechanism of interference remains unresolved. Attempts to quantify chicken type I interferon in the supernatant of infected cells were inconclusive (data not shown). Further studies are needed to elucidate the molecular basis of orthobornavirus superinfection exclusion / viral interference.

In summary, this study demonstrates that PaBV-4 exhibits marked host restriction *in vitro*, replicating efficiently in most avian but not mammalian cell lines, while the lack of replication in turkey embryo fibroblasts suggests that susceptibility may also differ among avian taxa. Despite serial propagation in cell culture for up to 24 passages, the viral genome remained relatively stable, with only limited genetic diversification and no evidence of host-specific molecular adaptation. Finally, persistent infection established a state of viral interference that markedly restricted superinfection by the related

orthobornavirus ABBV-1, while exerting only a transient inhibitory effect on the unrelated mononegavirus NDV.

Methods

Cells and passaging conditions. A total of ten cell lines were used in our study: 4 primary and 6 immortalized from avian and mammalian species. Primary cell lines were embryonic fibroblasts of chicken, Pekin duck, Canada goose, and domestic turkey (CEF, DEF, cGEF, and TEF, respectively), which were derived from embryonated eggs according to standard methods^{37 32}. Immortalized cell lines were purchased from American Type Culture Collection (ATCC) or obtained from colleagues, and included: embryonic fibroblasts of chicken (DF-1; ATCC#CRL-3586) and Pekin duck (CCL-141; ATCC#CCL-141), quail fibrosarcoma (QT-35; CVCL-4280), mouse glioma (CT-2A; Sigma Aldrich SCC194), baby hamster kidney (BHK-21; ATCC#CCL-10), and African green monkey kidney epithelial (Vero; ATCC#CCL-81) cells.

All cell lines were maintained in Dulbecco's Modified Eagle Media (DMEM, Cytiva Hyclone Laboratories, Logan, UT, USA) with 10% fetal bovine serum (FBS, Mediatech Inc. BBA JR Scientific, 1242 Commerce Ave, Woodland, CA, USA) and 1% Penicillin-Streptomycin (PS, Cytiva Hyclone Laboratories, Logan, UT, USA), and routinely incubated at 37°C with 10% CO₂. For CCL-141, a concentration of 15% FBS was used. Immortalized and primary cell lines were passaged at a 1:3 split every 3rd or 4th day, respectively, using 0.125% Trypsin diluted in 1x TryPLE (Life Technologies, NY, USA).

Viruses

PaBV-4 and ABBV-1 stock preparation. The original PaBV-4 virus was isolated from the liver and kidney of a rose-breasted parakeet (*Psittacula alexandrii*) and passaged 9 times in DEF cells²¹. The open reading frame of the N protein was sequenced to yield > 99% identity with other extant PaBV-4 sequences (GeneBank accession MZ018602).

This PaBV-4 isolate was then propagated in CCL-141 to produce a large volume of purified cell-free virus stock to be used in all subsequent experiments, as previously described (passages 10–15)^{13,22}. Briefly, 85% confluent CCL-141 in 100 mm cell dishes (ThermoFisher Scientific, Fair Lawn, NJ, USA) were inoculated with a small volume (100 µL) of initial virus isolate in full media (10 ml), and then routinely passaged 1:3 as described above. For stock production, PaBV-4 was harvested from CCL-141 during 6 passages (p10-15 since initial isolation) by lysing cells from approximately 390 dishes through a combination of osmotic shock and freeze-thaw, as follows. After washes with phosphate buffer saline (PBS), cell cultures were overlain with sterile Milli-Q water (5 ml / 100 mm dish) for one hour at room temperature (to promote osmotic lysis), frozen at -80°C, and thawed at room temperature. Upon thawing, a cell scraper was used to disrupt the cell monolayer. Water containing cell lysates was harvested from the dishes and pooled into 50 mL conical centrifuge tubes (ThermoFisher Scientific, Fair Lawn, NJ, USA), diluted with 10 X PBS to achieve a final 1 X PBS concentration, and then centrifuged at 3,000 x g for 10

minutes. The supernatant was collected, incubated with polyethylene glycol 8000 (PEG-8000) at a 1:3 ratio (PEG:supernatant) overnight at 4°C with constant rocking, and then pelleted by centrifugation at 4000 x g for 15 minutes. The pellet was resuspended with PBS at approximately 1:100 of the original supernatant's volume¹³. The concentrated stock from multiple batches was mixed, aliquoted, and stored at -80°C until further use. The stock's titer was quantified by limiting dilutions in CCL-141 seeded in 96-well plates, coupled with immunofluorescence (IFA) against PaBV-4 N protein conducted 4–5 days after infection¹³. The resulting tissue culture infectious dose 50% (TCID₅₀) titer was then converted to FFUs by multiplying by a 0.6951 factor³⁸. The stock for ABBV-1 was produced in a similar way using CCL-141 cells, as previously described.^{13,22}

Newcastle disease virus stock preparation. The non-virulent (lentogenic) recombinant Newcastle disease virus (NDV; avian orthoavulavirus-1) used in this study was cloned to contain an additional red fluorescent protein (RFP) gene cassette between the hemagglutinin-neuraminidase (HN) and polymerase (L) genes (NDV-RFP), as previously described³⁹. The virus was passaged in embryonated 9–11-day old specific pathogen free (SPF) chicken eggs (Canadian Food Inspection Agency [CFIA], Ottawa, Canada), and the allantoic fluid harvested to produce the virus stock that was titered by evaluating red fluorescence in Vero cells plated on 96-well plates, to obtain a TCID₅₀ titer converted to FFUs³⁸.

Growth kinetics of PaBV-4 in cell lines. To generate multi-step growth curves and characterize the replication kinetics of PaBV-4, approximately 1 x 10⁶ CEF, DEF, cGEF, TEF, DF-1, CCL-141, QT-35, CT-2A, BHK-21, and Vero were used to seed each well of a six-well plate and infected in triplicate through the addition of either 2 mL of maintenance media containing 2.18 x 10³ FFUs (MOI: 0.001) of purified PaBV-4 stock or PBS (sham inoculum), and left to incubate for one hour at room temperature on a rocker set to medium speed. The inoculum was then removed and replaced with fresh maintenance media, and cell cultures kept at 37°C and 10% CO₂. Passaging occurred every 4 days, with a third of the total cell count from each well (as determined by hemocytometer) being used for IFA to assess viral spread (see below), one third pelleted for RNA extraction and reverse-transcriptase quantitative PCR (RT-qPCR), and the remaining third used for propagation (**Fig. S2**). Cells were grown for 6 passages (p21–24 from tissue isolation; note, the exact number of passages at the end of the growth curve vary since the virus stock in CCL-141 was not all harvested at one point, but from staggered sets of plates during the 6 passages).

For IFA, cells from each passage and each well were let adhere in a 35 mm dish for 24 hrs and then assayed for immunoreactivity against PaBV-4 N protein. Briefly, for each well the number of fluorescent cells was counted on 3 images taken at 10 x magnification with the DAPI (4',6-diamidino-2-phenylindole; nuclear stain) and fluorescein isothiocyanate (FITC; N protein detection) channels using a fluorescent microscope (Zeiss Observer A1 AXIO Fluorescence Microscope; Carl Zeiss AG, Jena, Germany), for a total of 9 images per passage (considering triplicates). Counting of N-positive cells (i.e., presenting nuclear, or nuclear and cytoplasmic reactivity) over the total number of nuclei (determined by DAPI) was facilitated using the Analyze Particles tool of ImageJ (National Institute of Health; NIH). The amount of virus copy number in the cell pellet of each passage was assessed by RT-qPCR (see below).

Immunofluorescence. Immunofluorescence against PaBV-4 N protein was carried out to titer the PaBV-4 stock after concentration, and to assess the percentage of infected cells at each passage during the multi-step growth curves, using a previously established protocol²². Briefly, plated cells were washed twice with PBS and fixed with methanol-acetone (50:50) at -20°C for 20 minutes. After washes, cells were then blocked at room temperature for 1 hr in blocking buffer (PBS with 0.1% v / v Tween-20 [PBS-T] with 10% v / v goat serum), and incubated overnight at 4°C in blocking buffer containing 1 µg / mL of primary mono-specific rabbit antibody (custom-made, Pacific Immunology) directed against a linear epitope conserved in the carboxy-terminal portion (aa 332–354) of numerous avian orthobornaviruses (shared identity with PaBV-4, 84%)²². After three washes with PBS–T, cells were incubated at room temperature for 1 hr with PBS-T containing 1 µg / mL secondary goat-anti-rabbit conjugated with AlexaFluor488, making sure to avoid photobleaching in a dark room. Cells were then washed with PBS-T, counterstained with DAPI (1 µg / mL; diluted in PBS-T), and imaged. The same procedure was used to titer the ABBV-1 stock, as previously described²².

Quantification of PaBV-4, ABBV-1 and NDV-RFP RNA. To quantify the RNA copies of PaBV-4, ABBV-1, and NDV-RFP from cell lysates, an RT-qPCR assay was employed. Briefly, total cellular RNA was extracted from cell pellets with Omega Bio-Tek E.Z.N.A. Total RNA Kit II (Omega Bio-Tek, Inc., Norcross, GA, USA) following the manufacturer's protocol, and reverse transcribed and amplified using the Luna Universal Probe one-step RT-qPCR kit (NEB, Whitby, ON), with 100 ng of total RNA per reaction. For PaBV-4 and ABBV-1, primers and probes targeting the N gene were employed. PaBV-4: forward primer, 5'- TGC CAT TCG CCA CTC CGA TG -3'; reverse primer, 5'- TCG TGC TAG TTG GGC CTC CT -3'; probe, 5'-6-FAM/TGG TCC ATA /ZEN/AAC TCC TTG CCG GGA /3IABkFQ/ -3'; ABBV-1: forward primer, 5'-ATG CAC TTG CAC TCT TAG AC-3'; reverse primer, 5'-TCC CCA TAA AAC CTC CCA AC-3'; probe, 5'-6-FAM-CCC TGC CCG CAG AGA GAA ATT CCA T-BHQ-3'²². For NDV-RFP, primers and probes targeting the matrix (M) gene were employed: forward primer, 5'- AGT GAT GTG CTC GGA CCT TC -3'; reverse primer, 5'- CCT GAG GAG AGG CAT TTG CTA – 3'; probe, 5'- /56-FAM/TT CTC TAG C/ZEN/A GTG GGA CAG CCT GC/3IABkFQ/ -3'⁴⁰. Cycling was performed using a LightCycler 480 System (Roche Diagnostics Corporation, Indianapolis, Indiana, United States), with the following conditions: 55°C for 10 minutes for reverse transcription; 95°C for 1 minute for initial denaturation; 95°C for 10 seconds for denaturation; and 60°C for annealing and extension, for a total of 40 cycles. A cycle threshold (Ct) ≥ 35.0 was considered negative. For all viruses, RNA quantification was done by interpolating Ct values against a standard curve made of ten-fold serial dilutions of a gene Block with the target PCR sequence (one per virus). All primers, probes, and the gene blocks were sourced from IDT Integrated DNA Technologies (Coralville, IA, USA), except for the NDV gene block, which was sourced by GenScript (Piscataway, NJ, USA). The readout was virus RNA copies per 100 ng of total cellular RNA (the template amount loaded in each reaction).

Superinfection study with PaBV-4 and ABBV-1. The objective of this experiment was to determine whether persistent infection with PaBV-4 or ABBV-1 interferes with subsequent infection by the heterologous orthobornavirus or permits long-term co-persistence within the same cell population. Six-well tissue culture plates (Thermo Fisher Scientific, Waltham, MA, USA) were seeded with 5 × 10⁵ naïve,

PaBV-4-persistently infected, or ABBV-1-persistently infected QT-35 cells per well, in triplicate. QT-35 cells persistently infected with either PaBV-4 or ABBV-1 were generated as described above and serially passaged until persistent infection was established, as indicated by stable viral RNA concentrations measured by RT-qPCR.

Cells were challenged with the reciprocal orthobornavirus (MOI = 0.01) for 2 h in DMEM supplemented with 8% fetal bovine serum and 1% penicillin-streptomycin, resulting in the following five groups: naïve cells challenged with PaBV-4 or ABBV-1; PaBV-4-persistently infected cells challenged with ABBV-1; ABBV-1-persistently infected cells challenged with PaBV-4; simultaneous PaBV-4 and ABBV-1 infection (each MOI = 0.01); uninfected cells. Following adsorption, inocula were removed, fresh medium was added, and cells were maintained by serial passage (1:4 split) every 3–4 days for 7–8 weeks. At designated passages, cell pellets were collected and stored at – 80°C until RNA extraction. Each sample was subsequently analyzed by virus-specific RT-qPCR assays targeting both PaBV-4 and ABBV-1 to quantify the relative abundance of each virus.

RNAscope in situ hybridization. To visualize the distribution of PaBV-4 and ABBV-1 RNA during persistent infection and superinfection, cells collected at selected passages ($\sim 10^5$ cells) were pelleted, fixed in 10% neutral-buffered formalin, embedded in paraffin, and processed for RNAscope® *in situ* hybridization (Advanced Cell Diagnostics, Newark, CA, USA). Consecutive 5- μ m sections were hybridized with RNAscope probes V-PaBV-4-N (targeting base pairs 141–1121 of PaBV-4 N gene; GeneBank accession MZ018602) and V-ABBV-1-N (targeting base pairs 55–590 of ABBV-1 N gene; GenBank accession MK966418.1). Slides were processed using the RNAscope 2.5 HD Duplex Reagent Kit (Advanced Cell Diagnostics) according to the manufacturer's instructions, and using the HybEZ hybridization oven (Advanced Cell Diagnostics). Sections were counterstained with hematoxylin, mounted, and examined by brightfield microscopy. For samples with established persistent infection, PaBV-4- and ABBV-1-specific probes were applied to consecutive serial sections in a simplex assay rather than duplex assays to facilitate interpretation of the weaker signal in the presence of abundant RNA from the resident virus. Samples collected during simultaneous coinfection were analyzed using duplex assay because the two viruses produced more comparable signal intensities.

Superinfection study with NDV-RFP. To assess whether PaBV-4-persistently infected avian cell lines displayed superinfection resistance to NDV-RFP challenge at two inputs (MOIs = 0.01 and 1), a coinfection experiment was devised with QT-35, DF-1, and CCL-141. Only immortalized cell lines were adopted, due to ensuing senescence with the primary lines over repeated passages.

Nine wells of 6-well tissue culture plates (ThermoFisher Scientific, Fair Lawn, NJ, USA) were seeded with 5.0×10^5 persistently infected cells. After approximately 12 hrs, cells were absorbed for 1hr with NDV-RFP at MOI = 1 and 0.01 in 1 ml of infection media (DMEM supplemented with 2% FBS, 1% PS, and 100 [for DF-1 and CCL-141] or 125 [for QT-35] μ g / mL trypsin [Life Technologies, NY, USA]). The inoculum was then discarded, wells washed with 2 ml PBS at least three times, and cells overlain with infection media. At 0, 6, 24, and 30 hpi for MOI = 1, or at 0, 6, 24, 30, and 48 hpi for MOI = 0.01, 200 μ L of

supernatant were collected for NDV-RFP titration in DF-1 cells, and replaced with fresh media (see below). An additional 6 wells per cell line (i.e., two sets of three replicates), infected with MOI = 0.01 and treated in the same way, were used to collect cell pellets at 6 and 24 hpi for RNA extraction and quantification of RNA copy numbers of NDV-RFP and PaBV-4 (see below). Cell lines were imaged by brightfield microscopy at 0, 18, and 48 hpi by brightfield microscopy for the assessment of morphological differences. A set of controls were run in parallel for each cell line following the same layout and conditions.

Plate reader-assisted tissue culture infectious dose 50% assay. For the superinfection study, titrations of NDV-RFP from the supernatant were carried out by limiting dilutions in 96-well plates seeded with DF-1 in infection media, to obtain TCID₅₀ titers upon assessment of fluorescence as a proxy for infection between 4 and 5 days after infection. Assessment of well fluorescence was partly automated through the use of a plate reader as described elsewhere (PerkinElmer Inc., Waltham, Massachusetts, United States)⁴¹. Briefly, the plate reader was set to an excitation and emission value of 532 and 588 nm, respectively. For each well, a total of 5 reads were taken, which were then averaged to obtain the fluorescent intensity. As part of the optimization, the fluorescent signals of 576 wells (6, 96-well plates) seeded at confluence with DF-1 cells persistently infected with PaBV-4 but not NDV-RFP (i.e., negative for fluorescence) were obtained. A threshold for calling positive wells was determined as the average fluorescence value + 4* standard deviation calculated for all 576 wells. This threshold yielded approximately a 1.5% rate of false positives, and was applied to 18 TCID₅₀ test plates (spanning between 102.84 and 105.46 TCID₅₀ units of NDV -RFP) that were scored both automatically and visually. The automated and visual titer estimation had a Bland-Altman bias = 0.06, a 95% limit of agreement between - 0.26 and 0.38, and a Pearson correlation coefficient = 0.99 ($p < 0.0001$). Given this high level of agreement, quantification of NDV-RFP was automated for all the other samples.

Full-genome PaBV-4 sequencing after passages. To evaluate development of virus mutations upon continuous passaging in cell lines from different species, the full genome of PaBV-4 was sequenced at different stages after isolation. Briefly, the PaBV-4 full genome was sequenced from: the original tissue from which the virus was isolated (p0; pooled kidney and liver of the initially infected rose breasted parakeet), DEF cells where virus isolation was initially conducted (p5), CCL-141 sampled during stock production (p15), and in DF-1 (p21), QT-35 (p21), and CCL-141 (p24) at the end of the growth curve. From either cells or tissues, total cellular RNA was extracted from using the E.Z.N.A. RNA Kit II (Omega Bio-Tek, Norcross, GA, USA), following the manufacturer's standard protocol. Samples were checked for RNA integrity (threshold, RIN > 6.5) with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) before being submitted to Genome Quebec (Montreal, QC, Canada), where sample quality control (QC), library preparation, and Illumina sequencing were performed. Briefly, a secondary sample QC (RNA integrity and quantification) was performed with a LabChip GX Touch using DNA 5K/RNA Charge Variablt Assay LabChip (cat. CLS138949) and RNA assay reagent kit (CLS960010). Library preparation was then performed using 125 ng of total RNA and QIAseq FastSelect (Human/Mouse/Rat 96rxns) for depletion of rRNA. NEBNext RNA First Strand Synthesis and NEBNext Ultra Directional RNA Second Strand Synthesis Modules (New England BioLabs) were then used for cDNA synthesis. Libraries were

completed using the NEBNext Ultra II DNA Library Prep Kit for Illumina (New England BioLabs), quantified using the KAPA Library Quantification Kits - Complete kit (Universal) (Kapa Biosystems), and average fragment size determined on a Fragment Analyzer 5300 (Agilent). All adapters and PCR primers for library preparation were acquired from New England Biolabs. Finally, libraries were normalized, pooled, denatured in 0.02N NaOH, neutralized by pre-load buffer, and loaded on an Illumina NovaSeq X Plus 25B lane at 140pM, using the manufacturer's standard protocol. Runs were performed at 2x100 cycles (paired-end mode) using a phiX library mixed with libraries at a 1% level as a control. To demultiplex samples and generate FASTQ read data, the program BCL Convert 4.2.4 was used.

Determination of nucleotide variants. A bioinformatics workflow was implemented to evaluate presence of nucleotide polymorphism in the PaBV-4 full genomes sequenced from tissues derived from the different cell lines. One or two FASTQ files from each sample (pooled tissues, DEF-p5, CCL-141-p15, DF-1-p21, QT-35-p21, CCL-141-p24) were assessed for QC and adaptor sequence trimming using fastp (version 0.20.1). Reads from the tissues sample (i.e., unpassaged virus) were aligned using BWA MEM (version: 0.7.17-r1188) against the published reference genome of PaBV-4 (NC_030688.1), and samtools (samtools 1.20 Using htslib 1.20) was used to sort the aligned reads and generate a consensus sequence that was used as the new reference sequence for alignment and comparison with all other in vitro-derived samples. Using a similar process, all other FASTQ files were aligned to the new reference sequence using BWA MEM followed by sorting using samtools. For each sample, the read counts for each nucleotide position in the alignment was determined using bam-readcount [version: 1.0.1-unstable-8-c7c76e6 (commit c7c76e6)]. Nucleotide frequency (A, T, C, or G) was expressed as the percentage of the total reads for each position. A variant was called when the percentage of reads exhibiting a mutation surpassed 5% of the total read count and a minimum read depth of 20 per position.

Estimation of substitution rate. To estimate the apparent accumulation of nucleotide substitutions during serial passage, the tissue-derived PaBV-4 consensus genome was considered the ancestral reference sequence. Newly detected substitutions were identified sequentially along the experimental passage history (tissue-derived virus → DEF p5 → CCL-141 p15 → terminal passage in each cell line), and tallied at each passage. Variants that were lost during passaging were not counted as actual substitutions, as these were interpreted as frequency variants, rather than *de novo* mutational events. For each independently propagated lineage, the apparent substitution accumulation rate was calculated by dividing the cumulative number of newly detected substitutions by the product of the PaBV-4 genome length (8915 nucleotides) and the total number of passages elapsed since the tissue-derived virus. Rates were calculated separately for each lineage and summarized by averaging.

Data analysis. The growth of PaBV-4 in different cell lines was assessed by evaluating the percentage of infected (i.e., fluorescent) cells and virus RNA copies over passages. For the latter, the concentration of RNA copies per 100 ng of cellular RNA at each passage was normalized to the initial timepoint immediately after absorption (fold-change, FC), and log10 transformed. Differences in growth kinetics was assessed by comparing empirical areas under the curve (eAUC), calculated for both percentages of infected cells and RNA FC integrated for each replicated, and then averaged. This system was adopted

as it reflects the entire virus growth over the entire duration of the experiment, instead of comparing single values at different time points^{42 43}. The eAUCs were calculated using as baseline = 0, and excluding peaks < 10% of the maximum titer value. Differences in average eAUCs for each cell line were tested by one-way ANOVA with Tukey's test for multiple comparison.

For the orthobornavirus interference study, the RNA copy number of each virus at each time point was normalized to its average value at 3 dpi (beginning of the study), to generate fold-change values. The RNA copies for each virus were integrated over all time points separately for each replicate, to generate average eAUCs. Differences in average eAUCs for each experimental group were tested by one-way ANOVA with Tukey's test for multiple comparison.

The concentration of infectious NDV-RFP in the supernatant of QT-35, DF-1, and CCL-141 at 5 time points was assessed by limiting dilution in DF-1 cells (TCID₅₀ / ml), while the concentration of virus RNA copies was assessed in cell lysates at 6 and 24 hpi (RNA copies / 100 ng of total cellular RNA). Log₁₀-transformed NDV-RFP titers were compared using a two-way ANOVA with repeated measures fitting a full model (variables: cell line and passage) using the Geisser-Greenhouse's correction, and Tukey's test for multiple comparisons within each passage. Virus RNA copies at 6 and 21 hpi were log₁₀-transformed and compared by a two-way ANOVA (since RNA was extracted from different plates, measurements were considered independent) fitting a full model (variables: cell line and time) and using Tukey's test for multiple comparisons. The eAUP for NDV-RFP titers in the supernatant were calculated and tested as described above.

All tests were conducted as implemented using GraphPad Prism version 10.0.0 for Mac OS X (GraphPad Software, Boston, Massachusetts USA, www.graphpad.com). All tests were significant for test value (p or q) < 0.05.

Declarations

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Data availability. The data supporting this study are included in the article and its supplementary files. Original raw data are available from the corresponding author upon reasonable request. The sequence of the full PaBV-4 genome from the initial tissue is currently under submission.

Code availability. No custom code or software was generated for this study.

Competing interests. The authors declare no competing financial or non-financial interests.

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Figures

Figure 1

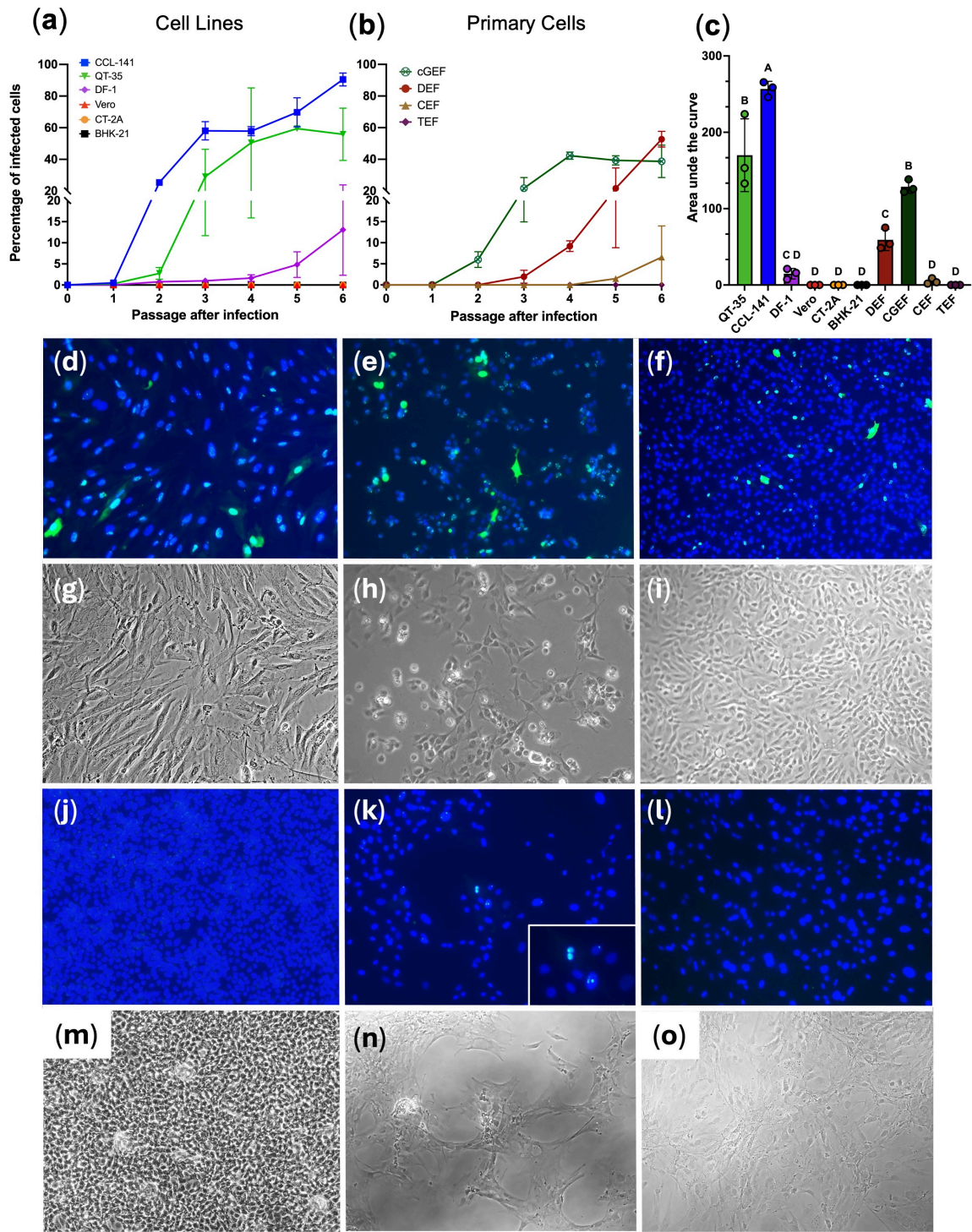


Figure 1

Immunofluorescence detection of PaBV-4 nucleoprotein in avian and mammalian cell cultures. The proportion of PaBV-4 nucleoprotein (N)-immunoreactive cells was determined by immunofluorescence assay (IFA) over six serial passages in immortalized avian and mammalian cell lines (a) and primary avian cell cultures (b). The cumulative proportion of infected cells over time was quantified as the empirical area under the curve (eAUC) (c). Data are presented as mean $\pm$ SD (n , 3), with individual replicates shown in (c). Different letters indicate statistically significant differences (one-way ANOVA with Tukey's multiple-comparison test, $p < 0.05$).

Figure 2

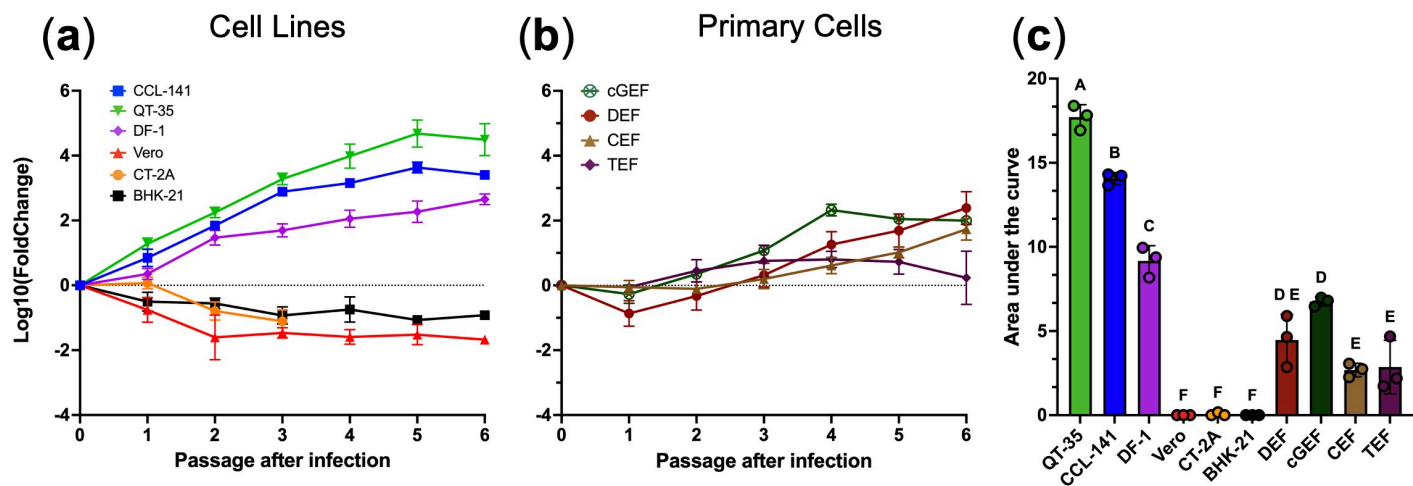
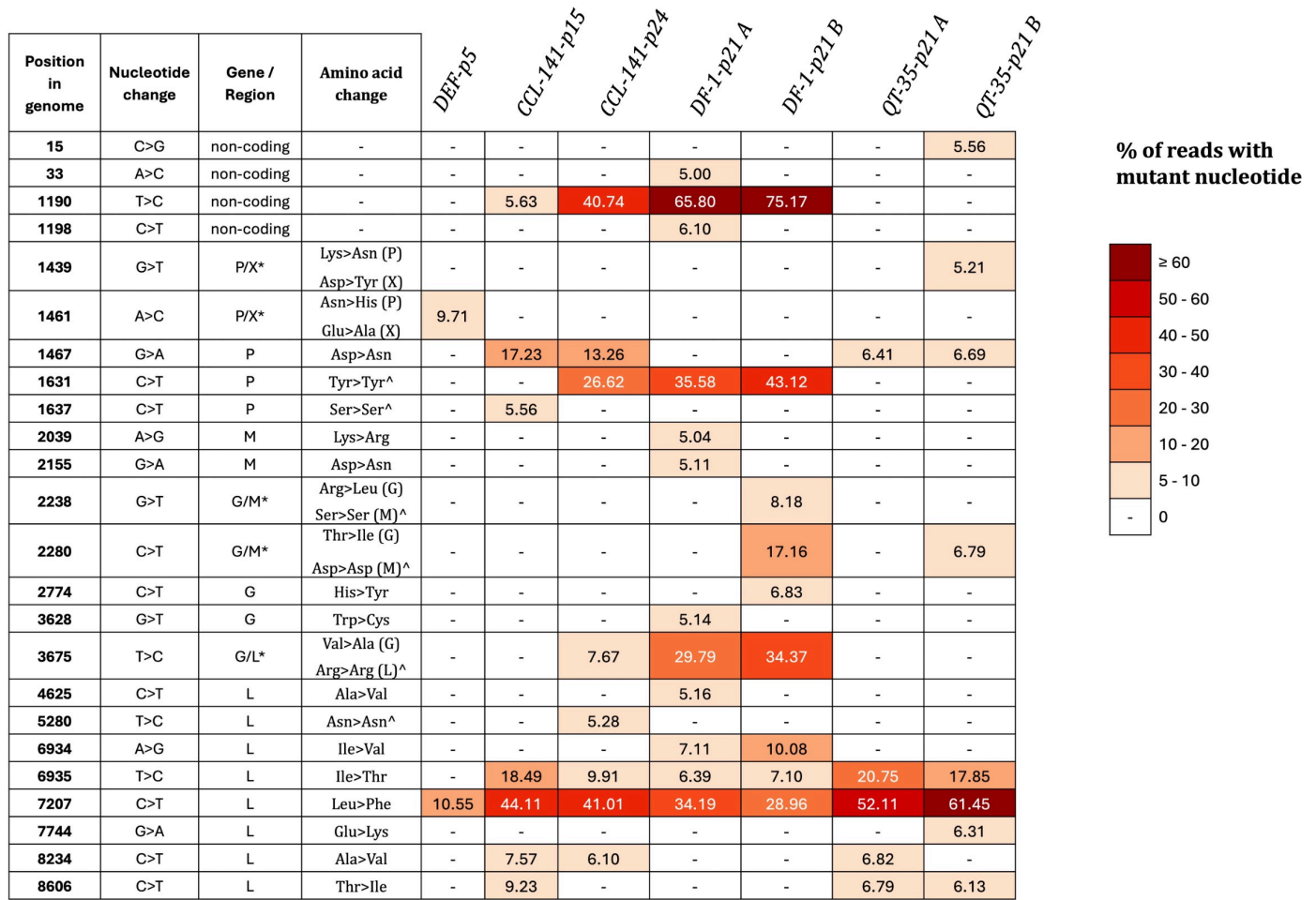


Figure 2

Quantification of PaBV-4 RNA during serial passage in avian and mammalian cell cultures. Viral RNA accumulation, expressed as log10 fold-change relative to passage 0, was quantified by RT-qPCR in immortalized avian and mammalian cell lines (a) and primary avian cell cultures (b). Viral RNA increased progressively in all permissive avian cells, with QT-35 exhibiting the greatest RNA accumulation, whereas mammalian cell lines showed no evidence of sustained viral replication. Integration of viral RNA fold change over time (eAUC) (c) demonstrated significantly greater RNA accumulation by the end of the study in QT-35, followed by CCL-141 and DF-1 cells. Data are presented as mean $\pm$ SD (n , 3), with scatterplot in c. Different letters indicate statistically significant differences (one-way ANOVA with Tukey's multiple-comparison test, $p < 0.05$).

Figure 3



*Positions in overlapping open reading frames (ORFs) that affect two proteins
 - = mutation not detected (< 5% or coverage < 20 reads)
 ^ = synonymous mutation

Figure 3

Nucleotide substitutions identified during serial passage of PaBV-4 in avian cell cultures. Heat map summarizing nucleotide substitutions detected by whole-genome sequencing following serial propagation of PaBV-4 in DEF, CCL-141, DF-1, and QT-35 cells. Columns represent the cell line at progressive passages (p) starting from original isolation from tissues, with A and B denoting biological replicates. Rows correspond to individual genomic positions. Substitutions were called (> 5% mutant reads, with sequence coverage > 20×) using the consensus sequence derived from tissues as the reference, with the percentage of sequencing reads containing the mutant nucleotide indicated numerically and represented by the corresponding color scale. Blank cells indicate that the substitution was not detected. Mutations located within overlapping open reading frames (ORFs) are marked with an asterisk (*) and the corresponding amino acid changes in both proteins are indicated. Synonymous substitutions are denoted by (^). Overall, 51 nucleotide substitutions were identified at 24 unique genomic positions, the majority of which were missense mutations. Although most substitutions

occurred at low to moderate frequencies and were lineage-specific, the recurrent C7207T substitution in the L gene was detected in all propagated lineages and increased to > 50% mutant reads in QT-35 p21, suggesting repeated selection during serial passage in cell culture.

Figure 4

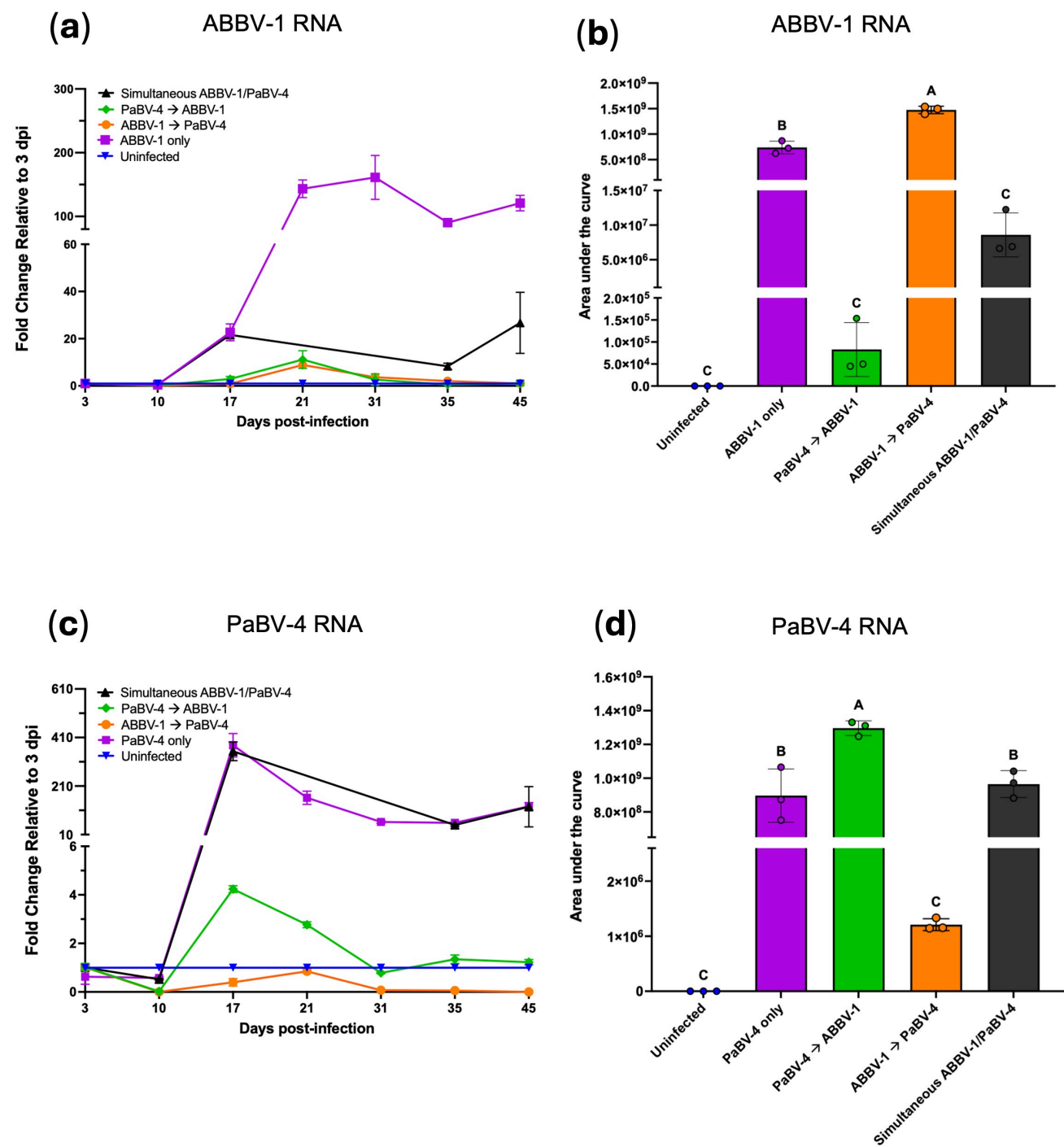


Figure 4

Reciprocal viral interference between PaBV-4 and ABBV-1 in persistently infected QT-35 cells. Viral RNA concentrations, expressed as fold change of RNA copies relative to 3 days post-infection (dpi), were quantified by RT-qPCR throughout reciprocal superinfection experiments using primers and probes specific for ABBV-1 (**a**) or PaBV-4 (**c**). QT-35 cells persistently infected with PaBV-4 were challenged with ABBV-1 (green), QT-35 cells persistently infected with ABBV-1 were challenged with PaBV-4 (orange), and naïve QT-35 cells were infected with either ABBV-1 or PaBV-4 alone (purple) or simultaneously with both viruses (black). Non-infected cells were kept as a control (blue). Persistent infection with either orthobornavirus markedly restricted replication of the superinfecting virus with no change on the RNA levels of the resident virus, whereas simultaneous coinfection permitted replication of both viruses, although PaBV-4 consistently accumulated to higher RNA concentrations than ABBV-1. Viral RNA copy numbers were integrated over the duration of the experiment to generate empirical areas under the curve (eAUC) for ABBV-1 (**b**) and PaBV-4 (**d**). Integration confirmed significantly reduced cumulative RNA levels of the superinfecting virus when persistent infection with the resident virus was already established (green bar in **b**; orange bar in **d**). Simultaneous coinfection resulted in intermediate cumulative RNA levels for ABBV-1 and PaBV-4, which for the latter were comparable to that observed following single-virus infection (black bar in **d**). In the labels, the virus preceding the arrow (à) represents the resident (persistent) virus and the virus following the arrow represents the challenge (superinfecting) virus. Data represent mean $\pm$ SD (n , 3), with superimposed single datapoints in **b** and **d**. Different letters indicate statistically significant differences among experimental groups (one-way ANOVA with Tukey's multiple-comparison test, $p < 0.05$).

Figure 5

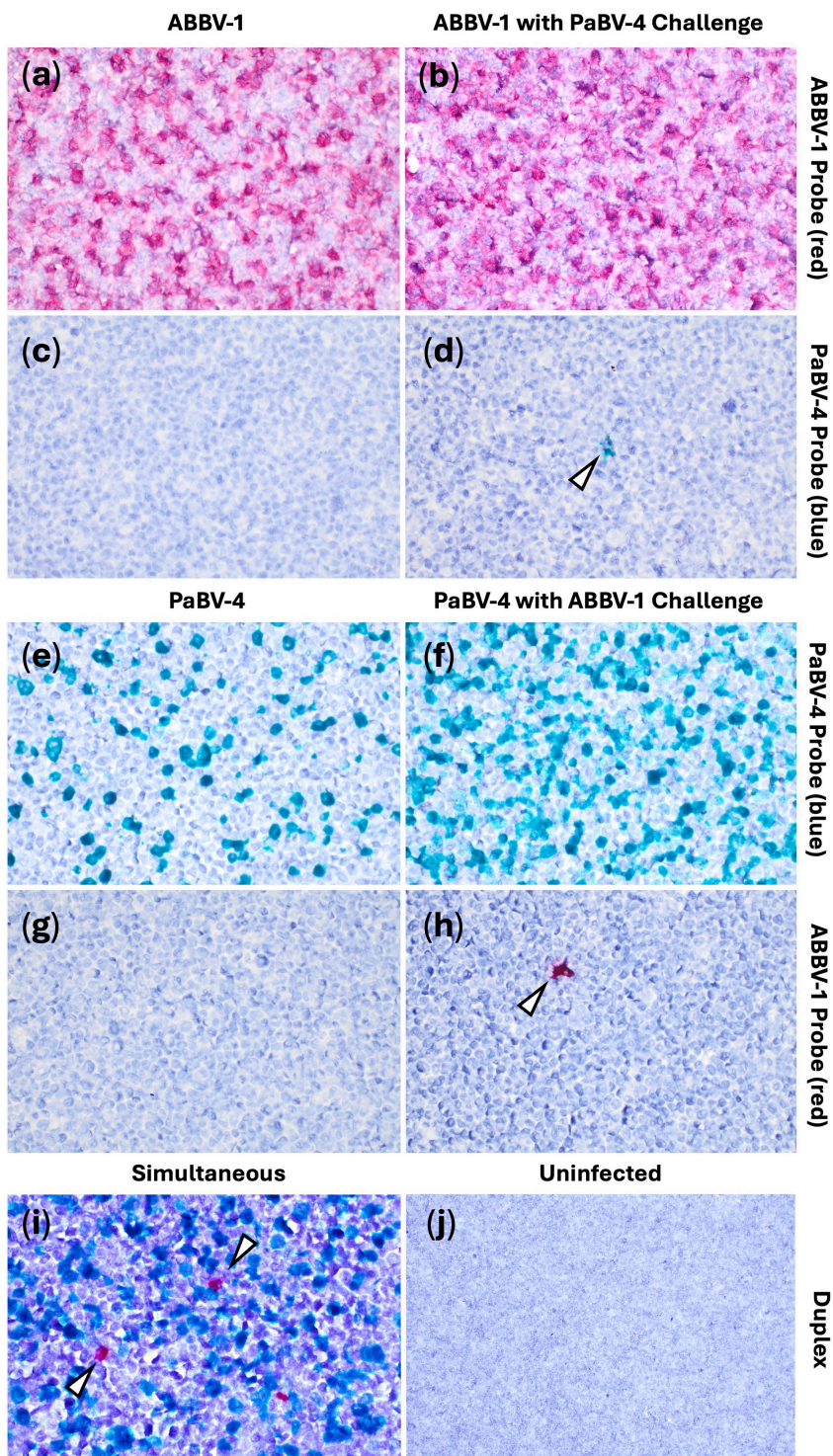


Figure 5

RNAscope *in situ* hybridization confirms reciprocal interference between PaBV-4 and ABBV-1.

Representative RNAscope *in situ* hybridization images of formalin-fixed, paraffin-embedded cell pellets from QT-35 that were either persistently infected, superinfected with reciprocal virus, or underwent simultaneous coinfection. ABBV-1 RNA was detected using a red chromogen and PaBV-4 RNA using a blue chromogen. Consecutive sections of QT-35 cells persistently infected with ABBV-1 alone (**a, c**) or

persistently infected with ABBV-1 and subsequently challenged with PaBV-4 (**b, d**) were hybridized separately with ABBV-1-specific (**a, b**) and PaBV-4-specific (**c, d**) probes. Similarly, consecutive sections of QT-35 cells persistently infected with PaBV-4 alone (**e, g**) or persistently infected with PaBV-4 and subsequently challenged with ABBV-1 (**f, h**) were hybridized separately with PaBV-4-specific (**e, f**) and ABBV-1-specific (**g, h**) probes. Abundant RNA from the resident virus was present in both unchallenged and challenged cultures, whereas only rare cells containing RNA from the superinfecting virus were detected following challenge (arrowheads in **d** and **h**). Duplex RNAscope of simultaneously coinfecting QT-35 cells demonstrated both PaBV-4 and ABBV-1 RNA within the same cell population, although ABBV-1-positive cells were comparatively fewer (arrowheads, **i**). No hybridization signal was observed in uninfected control cells (**j**).

Figure 6

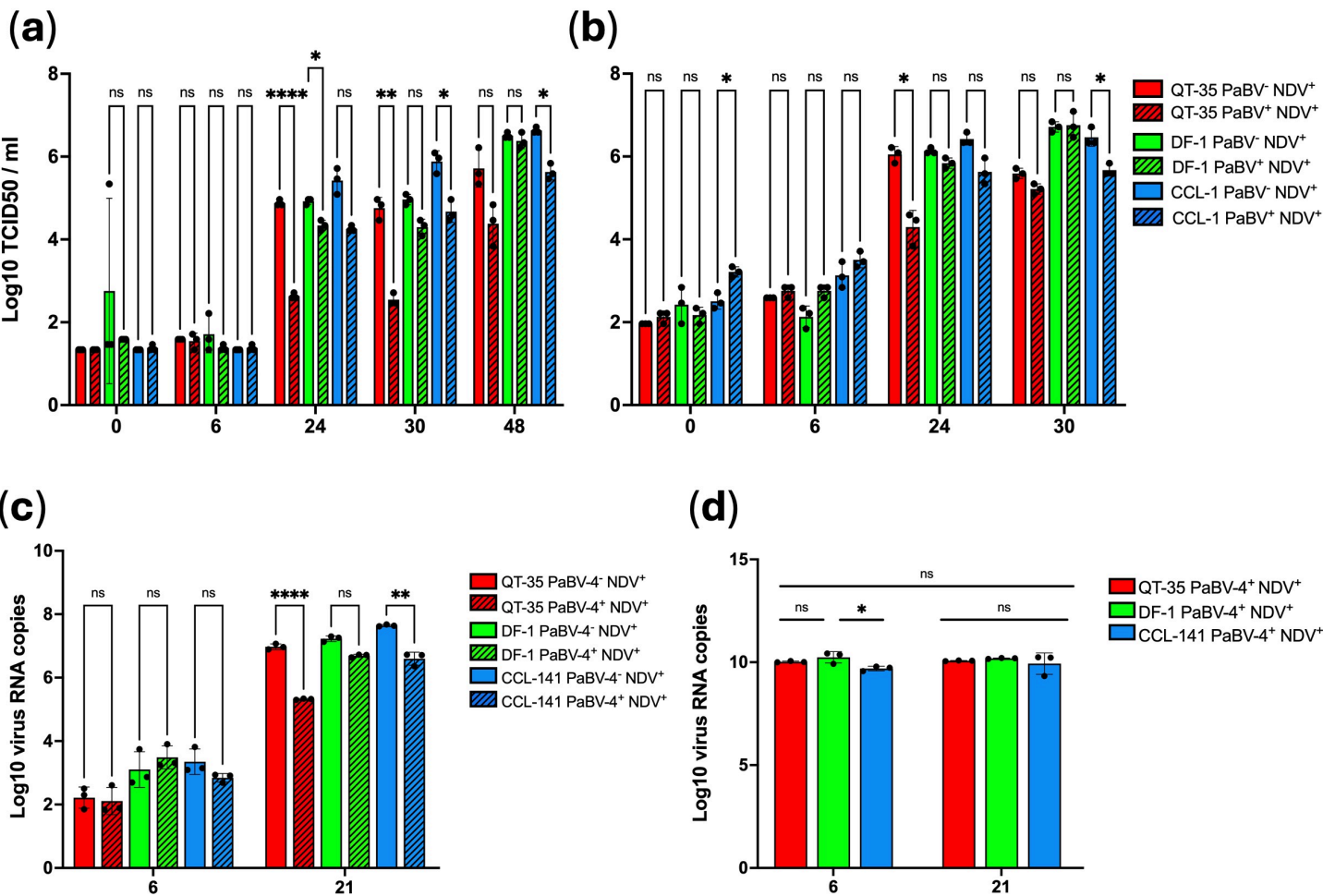


Figure 6

Multistep growth curve of NDV-RFP (multiplicity of infection (MOI) = 0.01 (**a, c**) or = 1 (**b**)) in QT-35, DF-1, and CCL-141 cell lines either persistently infected (oblique stripe pattern) with PaBV-4 or uninfected

(solid color). NDV titre was assessed in the supernatant by limiting dilution in DF-1 cells (reported as log₁₀ tissue culture infectious dose 50% (TCID₅₀) / ml) at different time points (**a**, **b**), or in the cell lysate by assessing the virus RNA copy number by RT-qPCR (reported as log₁₀ virus RNA copies / 100 ng of total cellular RNA) at 6 and 24 hours post infection (MOI = 0.01 only; **c**). Connectors indicate presence or absence of significant differences in NDV titres between the same PaBV-4-positive or negative cell line at the same time point. In (**d**), the concentration of PaBV-4 RNA virus copy number was assessed in the cell lysate at 6 and 24 hours post infection (reported as log₁₀ virus RNA copies / 100 ng of total cellular RNA). Connectors indicate presence or absence of significant differences in PaBV-4 RNA copies within the same time point, and across time points in the same cell line. For all graphs, represented are group mean +/- SD (*n*, 3). Statistical analysis was conducted with two-way ANOVA (full model fitted), with Sidak's multiple comparisons test (ns, not significant; * < 0.05; ** < 0.005; *** < 0.001; **** < 0.0001)

Supplementary Files

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